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Title: Method and Construct for inhibition of cell migration

FIELD OF THE INVENTION

The invention is in the field of therapeutic means and therapeutic methods for treatment of diseases in which cell migration and/or tissue remodeling occurs. Furthermore, the invention is in the field of biotechnology, in particular recombinant DNA technology and gene therapy.

BACKGROUND OF THE INVENTION

Migration of cells is an essential step in many physiological and pathological processes in which tissue remodeling occurs, such as tumor metastasis, wound healing, restenosis, angiogenesis or rheumatic arthritis. Migrating cells have to pass through the surrounding extracellular matrix. Limited proteolytic degradation of the components of the extracellular matrix is often seen during cell migration. To mediate this cell migration migrating cells produce, or recruit from their direct environment, proteolytic enzymes, such as plasminogen activators, metalloproteinases or elastases. Induction of cell migration e.g. during tumor metastasis or wound healing often correlates with the induction of the production of these enzymes.

Although the involvement of proteolytic enzymes in cell migration under pathophysiological conditions is well accepted, little attempts have been made to inhibit cell migration by inhibiting these proteolytic enzymes. A possible explanation for the limited use of protease inhibitors is the fact that these proteolytic enzymes are involved in many processes both pathological and physiological (including fibrinolysis, wound healing, growth factor activation etc.) and that inhibition of these protease systems by systemically applied protease inhibitors might have either strong side effects or may lead to a diffusion or clearance of the inhibitory compounds without having a strong effect on the local cell migration processes.

Another problem in the use of protease inhibitors to interfere in cell migration and tissue remodeling is that proteases mediating these processes can bind to receptors at the cell surface. In this way the proteolytic enzymes might be active locally in a pericellular microenvironment where they are protected against the action of the present inhibitors.

It has been disclosed that conjugates between the receptor binding part of u-PA (the aminoterminal fragment or ATF) and urinary trypsin inhibitor produced in vitro, inhibit migration of tumor cells in vitro (Kobayashi, Gotoh, Hirashima, Fujie, Sugino and Terao, Inhibitory effect of a conjugate between human urokinase and urinary trypsin inhibitor on tumor cell invasion in vitro. J. Biol. Chem. (1995) 270, 8361-8366). The conjugate these authors have used is made synthetically by mixing the isolated ATF fragments with the trypsin inhibitor.

Recently it has been disclosed that these conjugates also can be produced recombinantly (WO 97/25422).

A comparable construct consisting of a receptor binding u-PA fragment and its inhibitor PAI-2, to be produced recombinantly in yeast, has been described to inhibit tumor cell migration in WO 92/02553 (PCT/GB91/01322). In this way they have made a protease inhibitor that can bind to a specific receptor at the cell surface, the urokinase receptor, and this inhibitor can inhibit cell migration (in vitro). As to the use of these constructs in vivo, a problem is the application to and the prolonged presence at the site of desired action in vivo.

SUMMARY OF THE INVENTION

This invention provides a recombinant nucleic acid molecule comprising a vector useful for transfection or transduction of mammalian, e.g. human, cells, wherein said vector contains a nucleic acid insertion encoding an expressible hybrid polypeptide or protein which comprises a

domain with a binding function and a domain with an effector function. Herein, the domain with a binding function preferably comprises a receptor binding domain, and the domain with an effector function preferably has enzymatic activity, most preferably protease inhibitor activity.

Preferably, the receptor binding domain is selected from the group consisting of urokinase receptor binding domain of urokinase, receptor binding domain of epidermal growth factor, receptor associated protein that binds to LDL Receptor related protein (α_2-macroglobulin receptor) and VLDL Receptor.

Preferably, the domain with an effector function has protease inhibitor activity and comprises a protease inhibitor or active part thereof, said protease inhibitor being selected from the group consisting of (bovine) pancreatic trypsin inhibitor, (bovine) splenic trypsin inhibitor, urinary trypsin inhibitor, tissue inhibitor of matrix metalloproteinase 1, tissue inhibitor of matrix metalloproteinase 2, tissue inhibitor of matrix metallo-proteinase 3, and elastase inhibitor. The domain with an effector function may comprise (an active part of) two or more different protease inhibitors, or two or more copies of (an active part of) a protease inhibitor, or both.

Preferably, the vector is selected from the group consisting of viral and non-viral vectors useful for transfection or transduction of mammalian cells. The vector may be an adenovirus vector or a retrovirus vector useful for transfection or transduction of human cells.

The nucleic acid insertion encoding an expressible hybrid polypeptide or protein may be under the control of a cell- or tissue-specific promoter, such as an endothelial cell-specific promoter, or a vascular smooth muscle cell-specific promoter, or a liver-specific promoter.

This invention furthermore provides a process for preventing local proteolytic activity, extracellular matrix degradation, cell migration, cell invasion, or tissue

remodeling, comprising transfecting or transducing the cells involved or cells in their environment with a recombinant nucleic acid molecule as defined herein to obtain local expression of the hybrid polypeptide or protein encoded by said nucleic acid molecule.

Also, this invention provides a process for producing a hybrid polypeptide or protein which comprises a domain with a binding function and a domain with an effector function, comprising transfecting or transducing mammalian cells with a recombinant nucleic acid molecule as defined herein to obtain expression of the hybrid polypeptide or protein encoded by said nucleic acid molecule, and optionally recovering the hybrid polypeptide or protein produced.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically depicts the plasmids pCRII-uPA (left) and pCRII-ATF (right).

Figure 2 schematically depicts the plasmid pCRII-ATF-BPTI.

Figure 3 schematically depicts the plasmid pMAD5-ATF-BPTI.

Figure 4 shows the results of proteolytic matrix degradation experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of hybrid proteins in which a receptor binding domain is linked to a functional protein in order to induce a local action of this protein and to prevent systemic effects and/or diffusion. In particular this invention relates to such hybrid proteins that might be produced by a subset of cells as target cells after transfection or transduction with expression vectors. More specifically the invention relates to the use of such expression vectors, coding for hybrid proteins consisting of a receptor binding domain and a protease inhibitor domain, for the prevention of cell migration and tissue remodeling by

inhibition of proteases at the surface of migrating or invading cells.

The method and construct described in the present invention can be applied as therapy in diseases in which cell migration and/or tissue remodeling occurs.

The present invention addresses the solution of several negative aspects involved in the above described use of inhibitors according to the prior art:

- High local concentrations of hybrid proteins in the direct environment of the target cells can be obtained by production of the protein by the migrating cells themselves or cells in their immediate environment. This production can be induced by transfection or transduction of a certain subset of the cell population with a suitable vector encoding the hybrid protein. For this purpose, one may use recombinant adenoviral vectors, retroviral vectors, plasmid vectors, etc.
- Diffusion of the inhibitor and systemic side effects are prevented by binding the hybrid protein (by its receptor binding domain) to the cell surface of the target cell. Local expression of this hybrid protein also contributes to the reduction of systemic side effects, while the negative effect of diffusion of the protein is reduced by the production at the site where action is required. The local expression of the hybrid protein in specific subpopulations of cells, e.g. endothelial cells prone to migrate during angiogenesis, can be enhanced using cell type-specific or tissue-specific expression vectors, in which the expression of the protein is under control of a promoter with cell type-specific or tissue-specific regulatory elements.
- Binding of a protease inhibitor to a cell surface receptor can locate the inhibitor close to its molecular target, the cell surface bound proteolytic enzyme. Local inhibition of the proteolytic activity in the pericellular microenvironment may be achieved in this way.
- Binding of a protease inhibitor to a cell surface receptor for a proteolytic enzyme, such as the urokinase

receptor, may have an additional inhibitory effect. It prevents the binding of the proteolytic enzyme to its receptor, and thus strongly reduces the action of this enzyme as has been shown for blocking the binding of u-PA to its receptor which can strongly inhibit cell migration.

Hybrid proteins, for which the expression vectors (e.g. adenoviral or retroviral expression vectors) contain the encoding DNA sequences, might contain a region that binds to a cell surface receptor and that is not subsequently internalized. Receptor binding domains that can be used for this purpose are e.g. the u-PAR binding domain of urokinase plasminogen activator, the receptor binding domain of epidermal growth factor, the receptor associated protein (RAP) that binds to the LDL-R related protein (LRP), also called α_2-macroglobulin receptor, and the VLDL-receptor.

The inhibitor part of the encoded hybrid protein might consist of various protease inhibitors such as bovine pancreatic trypsin inhibitor, also called aprotinin or Trasylol®, other trypsin inhibitors such as urinary trypsin inhibitor, inhibitors for matrix-degrading metalloproteinases such the tissue inhibitors of metalloproteinases TIMP-1, TIMP-2 and TIMP-3, or variants thereof. Also inhibitors for other proteases like elastase are very suitable for being incorporated into the expression vector containing the DNA sequences encoding the hybrid proteins. Multiple copies of the DNA sequences encoding the functional protein part of the hybrid protein e.g. the inhibitor part, or combinations of different inhibitors or derivatives thereof might be incorporated into the DNA construct in the expression vector.

Another very attractive possibility would be to use such an expression vector encoding hybrid receptor binding protein to apply any functional protein that should exert its action in the local environment of the target cell, e.g. a protease involved in the activation of a growth factor or an other e.g. vasoregulatory component.

The action of the functional protein or protein domains of the hybrid protein is localized to the direct microenvironment of the target cells by binding of the receptor binding domain to a receptor at the surface of the target cells. Production of the hybrid protein in the direct environment of the target cells or even by the target cells themselves can be obtained by transfection or transduction of these cells by the use of expression vectors that might be based on a non-viral or an adeno- or retroviral vector system. Expression in specific cell or tissue types might be achieved by the use of specific promoter elements in the expression vectors. For example, for endothelial cell-specific expression (elements of) the promoter region of the human or murine pre-pro-endothelin gene (HUMEDN1B and MMU07982, respectively, GENBANK) can be used, for vascular smooth muscle cell-specific expression (elements of) the promoter region of the human vascular smooth muscle α-actin gene (HUMACTSA, GENBANK) can be used, and for liver-specific expression the promoter of the human albumin gene (HUMALBGC, GENBANK) can be used.

Local delivery of these vectors might be obtained using various commonly used methods, including catheters, topically applied gels containing the vectors or targeted delivery systems. For site-specific delivery to the vessel wall, e.g. to prevent restenosis and vessel wall remodeling after angioplasty, special catheters can be used. At the moment double balloon catheters, channeled balloon catheters, multiple needle catheters and balloon catheters coated with a vector containing a hydrogel are being used for vessel wall-specific delivery. Other ways to deliver the vectors directly into the vessel wall are the use of stents coated with vector containing coatings, topical application of vector containing hydrogels to the outside of the blood vessel or ex vivo delivery directly into the blood vessel during transplantation surgery. Ex vivo transduction of proliferating cells using retroviral vectors followed by a reinjection may

also be used to deliver the vector constructs at the site where their action is required.

The present application will be described hereinafter in further detail, while referring to the following examples. It is to be noted that these examples merely serve to illustrate the invention, not to restrict it.

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EXAMPLE 1

An expression plasmid encoding the aminoterminal fragment of urokinase plasminogen activator (u-PA), amino acids 1-138, hereafter referred to as ATF, can be constructed by deleting the DNA sequences encoding amino acids 139 till 401 in an expression plasmid for the full length u-PA using a polymerase chain reaction (PCR) with the following oligonucleotides: 5'-cccgggctttttccatctgcgcagtc-3' and 5'-agggtcaccaaggaagagaatggc-3'. After amplification by PCR the newly formed DNA fragment can be circularized by ligation to restore the circular character of the expression plasmid. In this way an expression plasmid encoding the ATF and the C terminal last 11 amino acid residues including the stop codon can be constructed.

The sequence of the thus formed DNA construct encoding the u-PA ATF fragment then is determined and compared with the predicted sequence as a control for possible mutations introduced during the construction procedure.

The construction pCRII-ATF from pCRII-uPA using PCR is shown in Figure 1. In figure 1, the area indicated between the lines was removed during the PCR amplification, resulting in the ATF plasmid. The plasmid pCRII-uPA is shown to the left, plasmid pCRII-ATF to the right.

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EXAMPLE 2

DNA fragments encoding amino acid residues 36-93 of bovine pancreatic trypsin inhibitor (BPTI) and the homologous amino acid residues of bovine spleen trypsin inhibitor (BSTI)

can be isolated by performing a PCR reaction on genomic DNA isolated for bovine aortic endothelial cells using the following oligonucleotides: 5'-*tcgcga*cctgacttctgcctagagc-3' covering nucleotides 2509 to 2533 (with modifications, indicated in *italics*, in the 5' region of the oligonucleotide to introduce a NruI site (underlined) for cloning purposes) of the BPTI gene according to the published sequence (GENBANK, BTBPTIG), and nucleotide 2442 to 2462 of the BSTI gene according to the published sequence (GENBANK, BTBSTIG) and 5'-*ggtcacc*cagggcccaatattaccacc-3' covering nucleotides 2677 to 2704 of the BPTI gene and 2610 to 2636 of the BSTI gene (modified in the indicated nucleotides (*italics*) to introduce a BstEII and a SspI site respectively (underlined)). The amplified DNA fragments then were cloned into an appropriate plasmid vector, pCRII or pUC13, and then the exact sequence of the amplified DNA fragments in the isolated clones was analyzed to differentiate between BPTI and BSTI which have a very high degree of homology.

EXAMPLE 3

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The DNA fragment encoding amino acids 1 to 207 of the human tissue inhibitor of metalloproteinase type 1 is isolated by performing a reverse transcriptase polymerase chain reaction on total RNA isolated from human foreskin fibroblasts by using the following oligonucleotides 5'-agagagacaccagagaacccaccat-3' covering nucleotides 41 to 65 of the human TIMP-1 cDNA (according to the sequence in GENBANK HSTIMPR) and 5'-tcattgtccggaagaaagatgggag-3' covering nucleotides 740 till 755. The amplified DNA fragment was cloned into an appropriate host vector, pUC13, and then the exact sequence of the amplified DNA fragment in the isolated clones was analyzed.

EXAMPLE 4

For construction of a recombinant adenovirus containing sequences encoding the ATF.BPTI hybrid protein,

this sequence is cloned in the adenoviral vector construction adapter and expression plasmid pMAD5. This plasmid contains part of the wildtype adenovirus type 5 DNA sequences, a Major Late Promoter (MLP), and a poly-adenylation (polyA) signal and can be used as either an expression vector or a shuttle vector to construct a recombinant adenovirus. This plasmid was derived from plasmid pMLP10 as follows. First pMLP10-lin was constructed by insertion of a synthetic DNA fragment with unique sites for the restriction endonucleases MluI, SplI, SnaB1, SpeI, AsuII and MunI into the HindIII site of pMLP10. Subsequently, the adenovirus BglII fragment spanning nt 3328 to 8914 of the Ad5 genome was inserted into the MunI site of pMLP10-lin. Finally, the SalI-BamHI fragment was deleted to inactivate the tetracycline resistance gene, resulting in plasmid pMAD5. To clone the ATF.BPTI sequence into the pMAD5 plasmid between the MLP promoter and the polyA signal the following strategy has been followed.

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Starting from a pCRII plasmid in which a 1373 base pair fragment of the uPA cDNA was cloned, a PCR reaction with the oligonucleotides 5'-*cccggg*cttttttccatctgcgcagtc-3' (SmaI site underlined and nucleotides changed in *italics*) and 5'-ag*ggtcacc*aaggaagagaatggc-3' (BstEII site underlined and nucleotides changed in *italics*) was performed as described in example 1 to make a pCRII-ATF plasmid (see figure 1). Subsequently this pCRII-ATF plasmid was digested with the restriction enzymes SmaI and BsteII. In parallel the pCRII-BPTI plasmid was digested with the restriction enzymes NruI and BsteII and the BPTI containing fragment was cloned into the pCRII-ATF plasmid (see figure 2). The construction pCRII-ATF-BPTI is shown in Fig. 2.

In a next step the ATF-BPTI sequence was cloned into pMAD5. This was done by digestion of the pCRII-ATF-BPTI plasmid with the restriction enzymes EcoRV and SpeI, isolation of the ATF-BPTI encoding DNA fragment and cloning of this fragment into the SnaBI and SpeI digested pMAD5

plasmid. The cloning was tested by restriction analysis and sequence analysis.

The pMAD5-ATF-BPTI shuttle vector for the construction of ATF-BPTI adenoviral vector is shown in Figure 3.

EXAMPLE 5

In a similar way as described in example 4 for pMAD5-ATF-BPTI a plasmid containing the BSTI-gene (pMAD5-ATF-BSTI) was constructed using the pCRII-BSTI plasmid instead of the pCRII-BPTI plasmid.

EXAMPLE 6

For construction of a recombinant adenovirus containing sequences encoding the ATF-TIMP1 hybrid protein, this sequence is cloned in the pMAD5 expression plasmid. This plasmid contains part of the wildtype adenovirus type 5 DNA sequences, a Major Late Promoter (MLP), and a polyadenylation (polyA) signal and can be used as either an expression vector or a shuttle vector to construct a recombinant adenovirus. To clone the ATF-TIMP1 sequence into the pMAD5 plasmid between the MLP promoter and the polyA signal, the following strategy has been followed.

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Starting from a pCRII plasmid in which a 1373 base pair fragment of the uPA cDNA was cloned, a PCR reaction with the oligonucleotides 5'-cccgggctttttccatctgcgcagtc-3' and 5'-agggtcaccaaggaagagaatggc-3' was performed as described in example 1 to make a pCRII-ATF plasmid (see figure 1). Subsequently this pCRII-ATF plasmid was digested with the restriction enzymes SmaI and BsteII.

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In parallel a fragment of the cDNA of TIMP1 in pUC13-TIMP1 encoding amino acid residues 1 to 184 of the mature protein, but lacking the signal peptide and the stop codon, was amplified using the following oligonucleotides 5'-tcgcgatgcacctgtgtcccacc-3' and 5'-ggtcacccaaatattggctatgtgggaccgcaggg-3'. These oligonucleotides contain recognition sites for the restriction

A enzymes NruI (first oligonucleotide, underlined) and BstEII and Ssp1 respectively (second oligonucleotide, underlined); these sites are needed for the cloning procedure.

The amplified DNA fragment was cloned into a pCRII vector and called pCRII-TIMP1. This vector was subsequently digested with the restriction enzymes NruI and BsteII and the TIMP1 containing DNA fragment was cloned into the pCRII-ATF plasmid (see figure 1).

In a next step the ATF-TIMP sequence was cloned into pMAD5. This was done by digestion of the pCRII-ATF-TIMP plasmid with the restriction enzymes EcoRV and SpeI, isolation of the ATF-TIMP encoding DNA fragment and cloning of this fragment into the SnabI and SpeI digested pMAD5 plasmid. The cloning was tested by restriction analysis and sequence analysis.

For construction of a recombinant adenovirus containing sequences encoding the ATF.TIMP1 hybrid protein, this sequence is cloned in the pMAD5 expression plasmid. This plasmid contains part of the wildtype adenovirus type 5 DNA sequences, a Major Late Promoter (MLP), and a poly-adenylation (polyA) signal and can be used as either an expression vector or a shuttle vector to construct a recombinant adenovirus. To clone the ATF.TIMP1 sequence into the pMAD5 plasmid between the MLP promoter and the polyA signal the following strategy has been followed.

sub A7 Starting from a pCRII plasmid in which a 1373 base pair fragment of the uPA cDNA was cloned, a PCR reaction with the oligonucleotides 5'-cccgggctttttccatctgcgcagtc-3' and 5'-agggtcaccaaggaagagaatggc-3' was performed as described in example 1 to make a pCRII-ATF plasmid (see figure 1). Subsequently on this pCRII-ATF plasmid a PCR reaction was performed using the oligonucleotides 5'-<u>aatatt</u>attgaacttcatcaagttcc-3' and 5'-gactctagagcaaaatgacaaccag-3' and the resulting DNA fragment was cloned into the pCRII cloning vector. In this way the signal peptide of u-PA is removed and a SspI

restriction enzyme recognition site is introduced (underlined). The resulting plasmid DNA is designated pCRIIATF*.

In parallel a fragment of the cDNA of TIMP1 in pUC13-TIMP1 encoding amino acid residues -23 to 184 of the TIMP-1 protein, including the signal peptide but lacking the stop codon, was amplified using the oligonucleotides 5'-agagagacaccagagaacccaccat-3' and 5'-aatattggctatctgggaccgcagg-3' containing a recognition site for the restriction enzyme Ssp1 (underlined) and cloned into a pCRII cloning vector. The resulting plasmid DNA is designated pCRII-TIMP1*.

This vector was subsequently digested with the restriction enzymes SspI and EcoRV and the TIMP1 containing DNA fragment was cloned into a EcoRV-SspI digested pCRII-ATF* plasmid. The resulting plasmid containing the TIMP-ATF DNA fragment was called pCRII-TIMP-ATF. In a next step, the TIMP-ATF sequence was cloned into pMAD5. This was done by digestion of the pCRII-TIMP-ATF plasmid with the restriction enzymes EcoRV and SpeI, isolation of the TIMP-ATF encoding DNA fragment and cloning of this fragment into the SnabI and SpeI digested pMAD5 plasmid. The cloning was tested by restriction analysis and sequence analysis.

EXAMPLE 7

Vectors encoding hybrid proteins containing multiple copies of the BPTI unit coupled to the ATF domain have been constructed. To construct these multiple BPTI vectors, the following strategy is followed.

The pMAD5-ATF-BPTI described in EXAMPLE 4 is digested with the restriction enzymes SspI and BstEII. In this way the vector is opened exactly in the open reading frame at the end of the BPTI sequence. The pCRII-BPTI plasmid described in EXAMPLE 2 is digested with NruI and BstEII resulting in a BPTI encoding DNA fragment with one blunt end (NruI). The fragment was then monodirectionally cloned into the SspI BstEII pMAD5-ATF-BPTI vector. The thus constructed

plasmid named pMAD5-ATF-BPTI-BPTI was used as a shuttle vector for the construction of recombinant adenoviruses.

This approach can be repeated multiple times to construct vectors containing multiple BPTI-domains.

EXAMPLE 8

A vector encoding a hybrid protein containing both a BPTI unit and a TIMP1 unit coupled to the ATF domain has been constructed. To construct this BPTI-TIMP vector, the following strategy is followed.

The pMAD5-ATF-BPTI described in EXAMPLE 4 is digested with the restriction enzymes SspI and BstEII. In this way the vector is opened right behind the BPTI sequence. The pCRII-TIMP plasmid described in EXAMPLE 6 is digested with NruI and BstEII resulting in a TIMP1 encoding DNA fragment with one blunt end. The fragment was then cloned into the SspI BstEII pMAD5-ATF-BPTI vector. The thus constructed plasmid named pMAD5-ATF-BPTI-TIMP was used as a shuttle vector for the construction of recombinant adenoviruses.

EXAMPLE 9

To monitor the production of a functional ATF-BPTI hybrid protein after transfection of cells with pMAD5 or transduction with a recombinant replication-deficient ATF-BPTI encoding adenovirus, the following tests have been performed.

- The production of the hybrid ATF-BPTI protein by CHO cells transfected with the pMAD5-ATF-BPTI was analyzed using a uPA ELISA that recognizes the ATF, the aminoterminal fragment of u-PA. Production of ATF-BPTI was clearly detectable both after transient transfection of CHO cells with the pMAD5-ATF-BPTI plasmid (50-100 ng/ml/24hrs) and after transduction with an ATF-BPTI encoding adenoviral vector (up to 1.5 μg/ml/24hrs).
- The cell culture media of CHO cells transduced with the ATF-BPTI adenovirus were analyzed using western blotting

techniques. After electrophoresis and blotting, parallel filters were analyzed with polyclonal antibodies against either u-PA or BPTI (raised against Trasylol®). In both filters a signal was detected at the same expected position at approximately 20kDa. This indicates that the protein produced indeed contains fragments of u-PA and BPTI, thus that the hybrid protein is produced.

- The function as an inhibitor of plasmin activity of the ATF-BPTI protein was first analyzed in solution using dilutions of the culture medium of ATF-BPTI virus infected CHO cells (approximately 1.8 μg/ml). They were incubated with plasmin (1 nM) and the activity of plasmin was measured using a chromogenic substrate. Trasylol® dilutions were used as control references. Plasmin inhibition by ATF-BPTI medium was very effective, diluting the medium 1000x (i.e. 100 nM ATF-BPTI) resulted in a 50% inhibition of the activity of 1 nM plasmin, a similar inhibition as was observed with 100 nM Trasylol®. Thus the activity of ATF-BPTI is comparable to that of commercially available Trasylol® (Bayer, Germany).

- The function of ATF-BPTI as an inhibitor for plasmin bound to the cell surface via the interaction of the ATF domain with the u-PA receptor (uPAR) was tested using mouse cell lines that are either or not transfected with the human uPA receptor gene. These cells were incubated for 6 hrs with diluted medium of the ATF-BPTI virus-infected CHO cells. Cell extracts were made of the uPAR-transfected cells and the parental mouse cells lacking the human uPAR. Parallel cultures underwent a short acid treatment (pH 3, 3 min) before the cell extracts were made. This treatment will remove any u-PA or ATF bound to the u-PA receptor. The cell extracts were incubated with 1nM plasmin and the plasmin activity was determined. Plasmin activity could only be inhibited by the cell extract of the u-PAR containing cell line. No inhibition of plasmin activity was observed in the cell extracts of parental cell line, lacking the u-PA receptor, and in the acid-treated u-PAR containing cell line.

This clearly indicates that ATF-BPTI can function as a u-PAR bound plasmin inhibitor.

TABLE 1

	% INHIBITION OF PLASMIN ACTIVITY			
cell line	uPAR transfected cell line		parental cell line	
acid treatment	-	+	-	+
% inhibition	93%	0%	0%	0%

EXAMPLE 10

Cell-specific expression of ATF-BPTI in endothelial cells e.g. to specifically inhibit the migration of endothelial cells during angiogenesis, is achieved by cloning sequences of the promoter of the human pre-pro-endothelin 1 gene (nucleotide 2180-3680 of HUMEDN1B (GENBANK)) in front of the ATF-BPTI encoding DNA in an adenoviral vector. In this way, highly endothelial cell-specific expression of the ATF-BPTI hybrid protein can be obtained.

EXAMPLE 11

Proteolytic degradation of the extracellular matrix is a key event in many cell migration and tissue remodeling processes. This proteolytic matrix degradation is often found to be mediated by urokinase-type plasminogen activation. In order to test whether infection with an ATF-BPTI encoding adenovirus can inhibit plasmin mediated extracellular matrix degradation, an experiment was performed using human synoviocytes. These cells were infected with an ATF-BPTI adenovirus while they were seeded on an ^{3}H-labeled extracellular matrix existing of bovine cartilage material. Profound inhibition of matrix degradation could be observed in the virus treated cells (figure 4) indicating that matrix

degradation can be inhibited by infecting cells with the ATF-BPTI encoding virus.

Figure 4 shows the degradation of cartilage matrix by human synoviocytes in the presence of plasminogen. Matrix is incubated with control medium (lane 1), synoviocytes (lane 2), synoviocytes infected with ATF-BPTI adenovirus (lane 3), and synoviocytes incubated with Trasylol®(100KIU/ml)(lane 4).

EXAMPLE 12

In the process of restenosis smooth muscle cell migration and vessel wall remodeling are key events in which plasmin mediated proteolysis of extracellular matrix components is involved. In vivo application of general plasmin inhibitors to interfere in this process may have systemic side effects. Application of a plasmin inhibitor to the surface of the migrating cells might prevent these side effects. Infection of the blood vessel wall with an ATF-BPTI adenovirus at a site where neointima formation can be expected, e.g. in a transplanted "coronary by-pass" graft, might be a ideal way to produce the ATF-BPTI locally, and thus inhibit plasmin activity in the direct surroundings of the migrating (smooth muscle) cells, resulting in a reduced neointima formation.

This principle was tested using human saphenous vein organ cultures, a model system in which neointima formation can be mimicked very realistically. In parallel cultures, either or not infected with an ATF-BPTI adenovirus, the neointima formation was analyzed after three and four weeks. In the untreated tissues a clear neointima formation could be observed. Profound inhibition of the neointima formation could be observed in the tissues treated with 10^{10} pfu/ml ATF-BPTI adenovirus.

Appendix

Description and Nucleotide sequence of the pMAD5-ATF-BPTI plasmid.

From	To	Description
1	184	adenovirus sequence 5'
184	447	adenovirus Major Late Promoter (MLP)
447	644	tripartite leader sequence (TPL)
685	1167	urokinase ATF sequence
1168	1353	bovine prancreas trypsin inhibitor sequence
1360	1443	urokinase 3' sequence (including stop codon)
1514	1615	sequence derived form pSP65 and LacZ
1616	1751	SV40 poly A signal
1752	7334	adenovirus sequence 3'
9831	8971	β-lactamase

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~~Nucleotide sequence:~~

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1     CATTTTCGCG GGAAAACTGA ATAAGAGGAA GTGAAATCTG AATAATTTTG TGTTACTCAT
61    AGCGCGTAAT ATTTGTCTAG GGCCGCGGGG ACTTTGACCG TTTACGTGGA GACTCGCCCA
121   GGTGTTTTTC TCAGGTGTTT TCCGCGTTCC GGGTCAAAGT TGGCGTTTTA TTATTATAGT
181   CAGCTGATCG AGCGGTGTTC CGCGGTCCTC CTCGTATAGA AACTCGGACC ACTCTGAGAC
241   GAAGGCTCGC GTCCAGGCCA GCACGAAGGA GGCTAAGTGG GAGGGGTAGC GGTCGTTGTC
301   CACTAGGGGG TCCACTCGCT CCAGGGTGTG AAGACACATG TCGCCCTCTT CGGCATCAAG
361   GAAGGTGATT GGTTTATAGG TGTAGGCCAC GTGACCGGGT GTTCCTGAAG GGGGGCTATA
421   AAAGGGGGTG GGGGCGCGTT CGTCCTCACT CTCTTCCGCA TCGCTGTCTG CGAGGGCCAG
481   CTGTTGGGGC TCGCGGTTGA GGACAAACTC TTCGCGGTCT TTCCAGTACT CTTGGATCGG
541   AAACCCGTCG GCCTCCGAAC GGTACTCCGC CACCGAGGGA CCTGAGCGAG TCCGCATCGA
601   CCGGATCGGA AAACCTCTCG AGAAAGGCGT CTAACCAGTC GCTGATCGAT AAGCTAGCTT
661   ACGCGTACAT CTGCAGAATT CGGCTTAACT CTAGACCATG AGAGCCCTGC TGGCGCGCCT
721   GCTTCTCTGC GTCCTGGTCG TGAGCGACTC CAAAGGCAGC AATGAACTTC ATCAAGTTCC
781   ATCGAACTGT GACTGTCTAA ATGGAGGAAC ATGTGTGTCC AACAAGTACT TCTCCAACAT
841   TCACTGGTGC AACTGCCCAA AGAAATTCGG AGGGCAGCAC TGTGAAATAG ATAAGTCAAA
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901   AACCTGCTAT GAGGGGAATG GTCACTTTTA CCGAGGAAAG GCCAGCACTG ACACCATGGG
961   CCGGCCCTGC CTGCCCTGGA ACTCTGCCAC TGTCCTTCAG CAAACGTACC ATGCCCACAG
1021  ATCTGATGCT CTTCAGCTGG GCCTGGGGAA ACATAATTAC TGCAGGAACC CAGACAACCG
1081  GAGGCGACCC TGGTGCTATG TGCAGGTGGG CCTAAAGCCG CTTGTCCAAG AGTGCATGGT
1141  GCATGACTGC GCAGATGGAA AAAAGCCCCG ACCTGACTTC TGCCTAGAGC CTCCATATAC
1201  GGGTCCCTGC AAGGCCAGAA TTATCAGATA CTTCTACAAC GCCAAGGCTG GGCTCTGCCA
1261  GACCTTTGTA TATGGCGGCT GCAGAGCTAA AAGAAACAAT TTCAAGAGCG CAGAGGACTG
1321  CATGAGGACC TGTGGTGGTA ATATTGGGCC CTGGGTCACC AAGGAAGAGA ATGGCCTGGC
1381  CCTCTGAGGG TCCCCAGGGA GGAAACGGGC ACCACCCGCT TTCTTGCTGG TTGTCATTTT
1441  TGCTCTAGAG TCAAGCCGAA TTCTGCAGAT ATCGTCCATT CCGACAGCAT CGCCAGTCAC
1501  TATGGCGTGC TGCTAGAGGA TCCCCGGGCG AGCTCGAATT CCAGCTGAGC GCCGGTCGCT
1561  ACCATTACCA GTTGGTCTGG TGTCAAAAAT AATAATAACC GGGCAGGGGG GATTCTGAAC
1621  TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT
1681  AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT
1741  CATGTCTGGA TCTGGAAGGT GCTGAGGTAC GATGAGACCC GCACCAGGTG CAGACCCTGC
1801  GAGTGTGGCG GTAAACATAT TAGGAACCAG CCTGTGATGC TGGATGTGAC CGAGGAGCTG
1861  AGGCCCGATC ACTTGGTGCT GGCCTGCACC CGCGCTGAGT TTGGCTCTAG CGATGAAGAT
1921  ACAGATTGAG GTACTGAAAT GTGTGGGCGT GGCTTAAGGG TGGGAAAGAA TATATAAGGT
1981  GGGGGTCTTA TGTAGTTTTG TATCTGTTTT GCAGCAGCCG CCGCCGCCAT GAGCACCAAC
2041  TCGTTTGATG GAAGCATTGT GAGCTCATAT TTGACAACGC GCATGCCCCC ATGGGCCGGG
2101  GTGCGTCAGA ATGTGATGGG CTCCAGCATT GATGGTCGCC CCGTCCTGCC CGCAAACTCT
2161  ACTACCTTGA CCTACGAGAC CGTGTCTGGA ACGCCGTTGG AGACTGCAGC CTCCGCCGCC
2221  GCTTCAGCCG CTGCAGCCAC CGCCCGCGGG ATTGTGACTG ACTTTGCTTT CCTGAGCCCG
2281  CTTGCAAGCA GTGCAGCTTC CCGTTCATCC GCCCGCGATG ACAAGTTGAC GGCTCTTTTG
2341  GCACAATTGG ATTCTTTGAC CCGGGAACTT AATGTCGTTT CTCAGCAGCT GTTGGATCTG
2401  CGCCAGCAGG TTTCTGCCCT GAAGGCTTCC TCCCCTCCCA ATGCGGTTTA AAACATAAAT
2461  AAAAAACCAG ACTCTGTTTG GATTTGGATC AAGCAAGTGT CTTGCTGTCT TTATTTAGGG
2521  GTTTTGCGCG CGCGGTAGGC CCGGGACCAG CGGTCTCGGT CGTTGAGGGT CCTGTGTATT
2581  TTTTCCAGGA CGTGGTAAAG GTGACTCTGG ATGTTCAGAT ACATGGGCAT AAGCCCGTCT
2641  CTGGGGTGGA GGTAGCACCA CTGCAGAGCT TCATGCTGCG GGGTGGTGTT GTAGATGATC
2701  CAGTCGTAGC AGGAGCGCTG GGCGTGGTGC CTAAAAATGT CTTTCAGTAG CAAGCTGATT
2761  GCCAGGGGCA GGCCCTTGGT GTAAGTGTTT ACAAAGCGGT TAAGCTGGGA TGGGTGCATA
2821  CGTGGGGATA TGAGATGCAT CTTGGACTGT ATTTTTAGGT TGGCTATGTT CCCAGCCATA
2881  TCCCTCCGGG GATTCATGTT GTGCAGAACC ACCAGCACAG TGTATCCGGT GCACTTGGGA
2941  AATTTGTCAT GTAGCTTAGA AGGAAATGCG TGGAAGAACT TGGAGACGCC CTTGTGACCT
3001  CCAAGATTTT CCATGCATTC GTCCATAATG ATGGCAATGG GCCCACGGGC GGCGGCCTGG
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3241  ATCATGTCTA CCTGCGGGGC GATGAAGAAA ACGGTTTCCG GGGTAGGGGA GATCAGCTGG
3301  GAAGAAAGCA GGTTCCTGAG CAGCTGCGAC TTACCGCAGC CGGTGGGCCC GTAAATCACA
3361  CCTATTACCG GGTGCAACTG GTAGTTAAGA GAGCTGCAGC TGCCGTCATC CCTGAGCAGG
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3481  AGGCGCTCGC CGCCCAGCGA TAGCAGTTCT TGCAAGGAAG CAAAGTTTTT CAACGGTTTG
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3841  CTTCGCCCTG CGCGTCGGCC AGGTAGCATT TGACCATGGT GTCATAGTCC AGCCCCTCCG
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